The pathology of pre-mRNA splicing

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The pathology of pre-mRNA splicing: a meeting in the Italian Alps

Workshop on Pre-mRNA Processing and Disease

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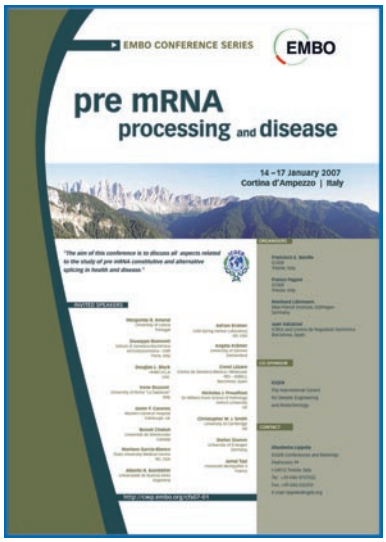

The EMBO workshop on Pre-mRNA Processing and Disease took place between 14 and 18 January 2007, in Cortina d' Ampezzo, Italy. It was linked to a satellite meeting focused on Mis-splicing and Disease, which received support from the European Network on Alternative Splicing (EURASNET). These workshops were organized by T. Baralle, R. Luhrmann, F. Pagani and J. Valcárcel.

Keywords: RNA processing; mRNA splicing; human disease

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Introduction

Gene expression is regulated extensively at the post-transcriptional level. The fundamental steps of eukaryotic RNA processing have been characterized in great detail, but knowledge of how the disruption of these processes contributes to human disease has only recently begun to emerge. This was the main theme of the EMBO Workshop on Pre-mRNA Processing and Disease held at Cortina d'Ampezzo, Italy, in the magnificent surroundings of the Dolomites.

Alterations in general splicing factors and human disease

Pre-messenger RNA (pre-mRNA) splicing is an essential step in eukaryotic gene expression. Intron sequences are excised from precursor mRNAs by the spliceosome, which is a large ribonucleoprotein complex composed of five small nuclear ribonucleoprotein particles (snRNPs)—U1, U2, U4, U5 and U6—and more than 100 non-snRNP splicing factors. Alternative splicing is an important mechanism for modulating gene expression and increases the proteomic complexity of a limited number of genes (reviewed by Matlin *et al*, 2005).

B. Chabot (Sherbrooke, Quebec, Canada) discussed the origin of introns in the genomes of higher eukaryotes. Bioinformatic analyses suggest that genomic duplication has been an important mode of intron gain in mammals (Zhuo *et al*, 2007). J. Beggs (Edinburgh, UK) presented evidence that in budding yeast the biogenesis of the U5 snRNP involves a cytoplasmic phase, and that the nuclear import of a U5-precursor complex is facilitated by a nuclear localization signal in PRP8P (pre-mRNA processing factor 8; PRPF8 in humans), a component of the U5 snRNP. Curiously, defects in several ubiquitously expressed genes encoding constitutive splicing factors, including PRPF31, PRPF3 and PRPF8, are associated with autosomal-dominant retinitis pigmentosa (adRP). Beggs showed that adRP-associated mutations in the carboxyl terminus of human PRPF8, when introduced into yeast PRP8P, affect its interaction with the U5 snRNP protein, Brr2, and cause a defect in U5 snRNP maturation. J. Wu (Chicago, IL, USA) discussed an immunoprecipitation microarray method to identify transcripts associated with the splicing factor *PRPF31*. Several such transcripts were identified, in which splicing was inhibited by a PRPF31 protein containing the identified adRP mutations. These results by Beggs and Wu highlight the importance of mutations in genes encoding general splicing factors in the aetiology of retinitis pigmentosa. Proposed models of how mutations in general splicing factors result in adRP pathogenesis are discussed in a recent review (see figure 3 in Mordes *et al*, 2006).

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Approximately 15% of mutations that cause genetic diseases affect pre-mRNA splicing, and they often target conserved splicing signals, including the 5' and 3' splice sites and the branch site (Krawczak *et al*, 1992). More recently, it has emerged that mutations in coding regions that were originally interpreted as silent single-nucleotide polymorphisms, nonsense mutations that introduce premature stop codons, or missense mutations that cause amino-acid changes, can instead affect pre-mRNA splicing. Such mutations target *cis*-acting elements that influence exon recognition, either by disrupting or by creating exonic splicing enhancers and/or silencers (reviewed by Pagani & Baralle, 2004).

T. Baralle (Trieste, Italy) described efforts to define new regulatory elements, known as composite exonic regulatory elements of splicing (CERES), in several disease-causing genes such as the cystic fibrosis gene, cystic fibrosis transmembrane conductance regulator (*CFTR*). These studies show that an exonic polymorphism cannot be ignored because it might affect the splicing process and lead to disease. This raises the issue of how functional splicing assays can be included in genotype screenings to distinguish between polymorphisms and pathogenic mutations.

Ser/Arg-rich proteins in cancer and development

A. Krainer (Cold Spring Harbor, NY, USA) reported that splicing factor, arginine/serine-rich 1 (*SFRS1*)—the gene encoding the prototypical serine/arginine-rich (SR) protein family member splicing factor 2/alternative splicing factor (SF2/ASF)—is a proto-oncogene. Various assays revealed that levels of SF2/ASF are elevated in different types of tumour. More importantly, SF2/ASF overexpression transforms immortal rodent cells and induces tumours in nude mice (Karni *et al*, 2007). Another link between SF2/ASF and oncogenesis was discussed by G. Biamonti (Pavia, Italy), who described how SF2/ASF controls cell motility by affecting alternative splicing of the *RON* (macrophage stimulating 1 receptor (c-met-related tyrosine kinase)) proto-oncogene, leading to the production of the oncogenic ΔRon isoform, which is found in breast and colon tumours (Ghigna *et al*, 2005).

J. Caceres (Edinburgh, UK) discussed the use of the ultraviolet crosslinking and immunoprecipitation (CLIP) method (Ule *et al*, 2005) to identify nuclear and cytoplasmic mRNA targets of heterogeneous ribonucleoprotein (hnRNP) A1 and SF2/ASF. hnRNP A1 was found to bind to, and be necessary for, the processing of a microRNA (miRNA) precursor, pre-miR-18a, which is part of the intronic oncogenic cluster miR-17-92. This finding underscores a new role for RNA-binding proteins as auxiliary factors that facilitate the processing of specific miRNAs (Guil & Caceres, 2007). An interesting link between SR protein expression and a developmental process was provided by J. Tazi (Montpellier, France). He reported that the SR protein B52/SRp55 controls developmentally regulated splicing of *eyeless*, a master gene of eye development in *Drosophila*. B52/SRp55 controls the alternative splicing of exon 2, which leads to the production of a protein isoform with an additional 60 amino acids and results in a small-eye phenotype. Conversely, the canonical *eyeless* isoform induces eye overgrowth. Thus, the splicing activity of B52/SRp55 controls eye organogenesis and size (Fic *et al*, 2007).

More RNA targets for splicing regulators

Identification of RNA targets of splicing regulators is crucial to understanding how alterations in splicing can result in human disease. A. Krämer (Geneva, Switzerland) also used the CLIP method to isolate RNA targets of the human splicing factor SF1 (also known as branch-point binding protein (BBP)) from HeLa cells. RNA interference (RNAi)-mediated depletion of SF1 affects the viability of human cells; however, it does not affect pre-mRNA splicing of several tested pre-mRNAs (Tanackovic & Kramer, 2005). This suggests that SF1 might have a kinetic role in splicing, rather than being an essential splicing factor. Analysis of the splicing of potential pre-mRNAs targets in SF1-depleted HeLa cells indicates that SF1 is involved in the splicing of only certain introns and modulates alternative splice-site choice. J. Stévenin (Illkirch, France) reported the identification of RNA targets of the testis-specific protein, RNA binding motif protein, Y-linked, family 1 (RBMY)—an hnRNP protein that interacts with several well-known splicing regulators, such as the SR proteins. *In vitro* selection (SELEX) experiments resulted in the identification of high-affinity binding targets for RBMY, consisting of RNA stem–loops capped by a pentaloop (Skrisovska *et al*, 2007).

The cross-regulation and functional redundancy between the splicing regulator polypyrimidine tract-binding protein (PTB) and its neuronal counterpart, nPTB, was discussed by D. Black (Los Angeles, CA, USA) and C. Smith (Cambridge, UK). PTB is an RNA-binding protein that acts widely as a splicing repressor (reviewed by Spellman *et al*, 2005). In the brain, the closely related paralogue, nPTB, is specifically expressed in post-mitotic neurons, whereas PTB is restricted to glial and other non-neuronal cells. Smith discussed the use of two-dimensional difference gel electrophoresis (2D-DiGE) to analyse the proteome of HeLa cells after RNAi knockdown of PTB. He observed an increase in nPTB—usually restricted to the neurons—which resulted from decreased skipping of nPTB exon 10, a splicing pathway that leads to nonsense-mediated decay (NMD). Simultaneous knockdown of both PTB and nPTB led to large changes in alternative splicing of several model pre-mRNAs, and numerous proteomic changes were accordingly observed using 2D-DiGE. Black showed that the splicing of a large group of exons is controlled during neuronal development by a switch in expression between PTB and nPTB, which display a mutually exclusive pattern of expression in the brain. The use of splicing-sensitive microarrays to identify different sets of exons regulated by PTB, nPTB or both proteins, revealed that the splicing of these exon sets is altered during neuronal differentiation, as predicted from the observed changes in PTB and nPTB expression (Fig 1). Therefore, the post-transcriptional switch from PTB to nPTB induces a widespread alternative-splicing programme during neuronal development (Boutz *et al*, 2007).

J. Ule (Cambridge, UK) discussed biochemical and computational approaches to derive an RNA map describing the rules by which Nova, a neuron-specific RNA-binding protein, regulates alternative splicing. The RNA map relates the position of Nova-binding sites (YCAY clusters) in a pre-mRNA to the outcome of splicing, and is able to predict the action of Nova (Ule *et al*, 2006).

M. Carmo-Fonseca (Lisbon, Portugal) described microarray studies analysing the expression levels of 181 splicing-related genes across different tissues and biological processes. The genes that were specifically up- or downregulated in a particular tissue included those for well-known RNA-binding proteins, such as members of the hnRNP and SR protein families, SR protein kinases, DEAD-box RNA helicases and tissue-specific splicing regulators, and also several core snRNP components. Robust signatures were associated with testis and whole brain, which are two organs with a high percentage of expressed genes that undergo alternative splicing.

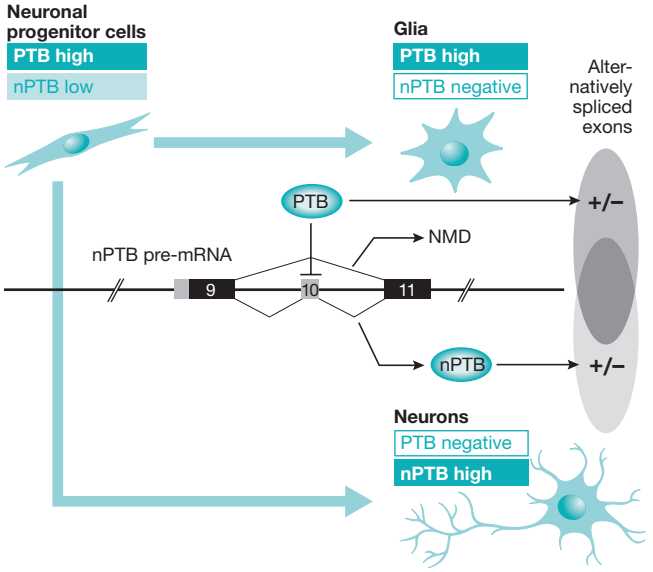

Fig 1 | Mutually exclusive pattern of expression of PTB and nPTB in the brain. PTB and nPTB have different positive and negative effects on the splicing of many exons, some of which overlap between the two factors (represented by the Venn diagram on the right). Figure kindly provided by P. Boutz, P. Stoilov and D. Black. NMD, nonsense-mediated decay; PTB, polypyrimidine tract-binding protein; nPTB, neuronal PTB.

A. Bindereif (Giessen, Germany) showed that a CA-rich element in intron 13 of the nitric oxide synthase gene acts as a length-dependent splicing enhancer that binds to hnRNP L. Exon microarray analysis identified genes in which hnRNP L acts either as an activator or as a repressor of various pre-mRNA processing events, including intron retention, exon skipping and poly(A)-site selection.

The *Drosophila* splicing regulator Sex-lethal (SXL) is expressed exclusively in female flies and regulates female-specific alternative splicing of at least three target genes: transformer (*tra*), male-specific-lethal 2 (*msl-2*) and *sxl* itself (reviewed by Forch & Valcárcel, 2003). J. Valcárcel (Barcelona, Spain) described microarray studies in flies that revealed widespread sex-specific changes in alternative splicing when comparing approximately 3,000 alternatively spliced genes in males and females. Several of these genes with sex-specific alternative splicing contain SXL-binding sites. Expression of SXL in male S2 cells resulted in partial recapitulation of the changes observed in the microarray studies.

Coupling and connections

Several talks at the meeting provided information on the connections between transcription and splicing. The transcription factor CA150—also known as transcription elongation regulator 1 (TCERG1)—localizes to nuclear speckles and is associated with factors involved in transcriptional elongation and splicing (Sanchez-Alvarez *et al*, 2006). M. García-Blanco (Durham, NC, USA) described a small interfering RNA (siRNA)-mediated CA150 knockdown approach followed by gene expression analysis to identify its cellular targets. He identified high-confidence CA150 targets, and chromatin immunoprecipitation (ChIP) analysis revealed CA150 occupancy of target gene loci, suggesting a direct effect on gene expression. C. Suñé (Granada, Spain) described the presence of CA150 in transcription pre-initiation and elongation complexes isolated *in vitro*, which should allow a biochemical understanding of how the interactions that occur during elongation affect alternative splicing events.

N. Proudfoot (Oxford, UK) extended the evidence for the formation of loops that join together the promoter and the 3' end of genes (G-loops), originally found in yeast (O'Sullivan *et al*, 2004), to mammalian cells. J. Kjems (Aarhus, Denmark) provided evidence that splicing events in human immunodeficiency virus-1 (HIV-1) can feed back and affect transcription of the viral genes. The failure of U1 snRNA to stably recognize a mutated 5' splice site inhibits transcriptional initiation by decreasing the recruitment of the general transcription factor TFIIH and of the TATA-binding protein. A. Kornblihtt (Buenos Aires, Argentina) presented two non-exclusive mechanisms that couple polymerase (pol) II transcription with alternative splicing. In the kinetic-coupling mechanism, the rate of pol II elongation determines a time period of opportunity for the splicing machinery to recognize and differentially use nascent splice sites. This mode was confirmed by using a slow mutant of pol II that promotes the inclusion of a cassette exon into the mature mRNA. Fluorescence recovery after photobleaching (FRAP) experiments, performed in collaboration with E. Bertrand (Montpellier, France), confirmed that the pol II mutant is indeed slow *in vivo*. In the recruitment-coupling mechanism, the C-terminal domain of pol II seems to have a crucial role by mediating an inhibitory effect of the SR protein SRp20 on the inclusion of a cassette exon in an elongation-independent manner (de la Mata & Kornblihtt, 2006).

Towards corrective therapy

Spinal muscular atrophy is a neurodegenerative disorder caused by the deletion or mutation of the survival-of-motor-neuron gene (*SMN1*). All affected individuals carry a nearly identical *SMN2* gene paralogue, which differs by a C to T transition in exon 7 that results in the skipping of this exon. Several strategies have been pursued to increase the extent of *SMN2* exon 7 inclusion. J. Marquis (Bern, Switzerland) described the use of bifunctional antisense molecules that tether SF2/ASF-binding sites to exon 7 through the antisense moiety (Skordis *et al*, 2003). To express these bifunctional RNAs in cells, Marquis and D. Schumperli (Bern, Switzerland) used vectors based on U7 snRNP. By changing the antisense sequences they targeted different regions of exon 7, to maximize the extent of its inclusion.

S. Stamm (Erlangen, Germany) addressed the connections between alternative splicing and signal transduction pathways by defining the roles of protein phosphatase 1 (PP1). This enzyme binds to a phylogenetically conserved RVDF sequence in the splicing factor transformer2-β1(tra2-β1), which is also present in eight other proteins, including SF2/ASF and SRp30c. PP1 dephosphorylates these proteins and influences their activities in splice-site selection. In particular, inhibition of PP1 promotes inclusion of *SMN2* exon 7, which suggests that reducing PP1 activity could be a new therapeutic strategy to treat spinal muscular atrophy.

Duchenne muscular dystrophy is an X-linked myopathy caused by deletions and point mutations in the dystrophin gene, some of which induce premature termination codons giving rise to NMD. Importantly, internal in-frame deletions in the dystrophin protein can be tolerated as they produce only mild myopathic symptoms; therefore, inducing exon skipping can be used as a therapeutic approach.

I. Bozzoni (Rome, Italy) showed that *in vivo* stable expression of constructs encoding the spliceosomal U1 snRNA—modified such that its 5' terminus is replaced by sequences complementary to the splice sites of the exon to be skipped—proved successful in rescuing both dystrophin synthesis and muscle function in the mdx mouse model of Duchenne muscular dystrophy (Denti *et al*, 2006).

C. Branlant (Nancy, France) described how the repressor protein hnRNP A1 competes with the SR proteins SRp40 and SC35, but not with SF2/ASF, for binding to selected regions of HIV-1 RNA to regulate splice-site selection in the generation of viral mature mRNAs. Conversely, M. Caputi (Boca Raton, FL, USA) described how members of the hnRNP H family of proteins assemble in a multiple complex on the HIV-1 genome to correctly regulate viral replication, splicing and the export of more than 40 viral mature mRNAs.

Connections to the clinic

The fibronectin gene undergoes extensive alternative splicing in three regions, generating up to 20 variants in humans. In particular, the alternatively spliced exon extra-domain A (EDA, also referred as EDI) has been extensively studied. E. White (Ann Arbor, MI, USA) reported that the EDA isoform is necessary for the development of pulmonary fibrosis, inducing the differentiation of myofibroblasts by an integrin-dependent mechanism. He reported that the phosphatase and tensin homologue (PTEN) signalling pathway blocks EDA exon inclusion through an unknown mechanism and prevents lung fibrosis, and that, conversely, patients with pulmonary fibrosis show decreased PTEN expression and increased EDA inclusion.

One of the aims in understanding global changes in alternative splicing associated with disease or triggered by external signals is to develop robust microarray systems that detect changes in mRNA isoform abundance with high sensitivity and reproducibility. A. Watkins (High Wycombe, UK) from Affymetrix presented both the system and the software developed by this company. In contrast to other systems that are based on exon–exon junction oligonucleotide probes, their alternative-splicing ChIP relies on exon-specific oligonucleotide probes.

R. Tupler (Modena, Italy) described the identification of *FRG1* overexpression as the cause of facioscapulohumeral muscular dystrophy (FSHD). FSHD patients carry deletions of several repeats that act as silencers of transcription of the *FRG1* gene. This results in overexpression of *FRG1*, which encodes a spliceosome-associated protein. A transgenic-mouse model overexpressing *FRG1* in skeletal muscle recapitulated the muscular dystrophy phenotype (Gabellini *et al*, 2006). Interestingly, specific pre-mRNAs undergo aberrant alternative splicing in the muscle tissue of FRG1 transgenic mice and also in FSHD patients.

Two talks dealt with splicing defects in membrane channels that cause 'channelopathies'. L. Crotti (Pavia, Italy) described mutations affecting splicing of the *HERG* (potassium voltage-gated channel, subfamily H (eag-related), member 2) and *SCN5A* (sodium channel, voltage-gated, type V, α-subunit) genes that cause Long QT syndrome, which is an electrical disorder responsible for sudden cardiac death. G. Woods (Cambridge, UK) found that mutations generating new splice variants in the α-subunit of the voltage-gated sodium channel SCN9A cause congenital insensitivity to pain (Cox *et al*, 2006). C. Lazaro (Barcelona, Spain) and D. Baralle (Cambridge, UK) discussed the importance of RNA analysis in the identification of the molecular defects associated with mutations in the neurofibromin 1 (*NF1*) gene that cause type-I neurofibromatosis. Lazaro presented a study of 374 *NF1* mutations, which revealed that 44% could be attributed to splicing defects. Baralle showed how a single point mutation in *NF1* intron 31 generates a new 3' splice site leading to the inclusion of a cryptic exon in the mature mRNA. By using mutant minigenes transfected into mammalian cells in culture, she defined the *cis*- and *trans*-acting determinants of the pathological exonization within intron 31.

F. Pagani (Trieste, Italy) studied a mutation that results in the expansion of a GAA triplet in frataxin intron 1, which causes Friedreich ataxia. By using a hybrid-minigene assay, he showed that the repeat expansion does not affect transcription; however, it induces aberrant splicing in a position-dependent manner, producing both exon-skipping and intron-retention forms of the mRNA. M. Amaral (Lisbon, Portugal) described mutations in the *CFTR* gene that cause cystic fibrosis by generating premature stop codons and triggering NMD. She proposed the use of aminoglycoside antibiotics that inhibit NMD as a therapeutic approach. I. Vorechovsky (Southampton, UK) addressed the links between splicing and complex trait diseases—that is, diseases caused by changes in multiple genes and that have a clear environmental component—such as type 1 diabetes and juvenile obesity, which are influenced by the susceptibility locus insulin-dependent diabetes mellitus 2 (*IDDM2*) comprising the insulin gene. He showed examples of single-nucleotide polymorphisms in intron 1 of the insulin gene that generate new splicing isoforms and are associated with type 1 diabetes.

Perspectives

The meeting in Cortina d'Ampezzo provided numerous examples of mutations in *cis*-acting sequences and *trans*-acting factors that affect pre-mRNA splicing of genes important for human disease. Interestingly, it became evident that mutations in genes encoding general splicing factors might cause specific human diseases, such as adRP and spinal muscular atrophy, instead of producing pleiotropic defects. Another important concept emerging from RNA analysis is that mutations in coding regions should not necessarily be assumed to be silent single-nucleotide polymorphisms or the cause of single amino-acid changes, but could instead markedly affect splicing and lead to human disease; this should be taken into account during genotype screenings. More importantly, there is now evidence that the accumulated basic knowledge is slowly but steadily translating into molecular therapies designed to correct defective pre-mRNA processing.

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